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ACUTE INTERMITTENT NICOTINE TREATMENT PRODUCES REGIONAL INCREASES OF BASIC FIBROBLAST GROWTH FACTOR MESSENGER RNA AND PROTEIN IN THE TEL- AND DIENCEPHALON OF THE RAT

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Abstract—Several findings show a neuroprotective effect of nicotine treatment in different experimental models, and a negative correlation has been observed between cigarette smoking and the incidence of Parkinson's disease. It seems possible that nicotine may in part exert its neuroprotective actions by favouring the synthesis of neurotrophic factors. The aim of this study was to determine whether the nicotine treatment could be associated with the induction of a neurotrophic factor in brain regions with nicotinic receptors. Thus, we analysed by *in situ* hybridization and RNAse protection assay the effects of (−)nicotine on basic fibroblast growth factor messenger RNA and by immunocytochemistry fibroblast growth factor-2 protein in the tel- and diencephalon of rats following single or acute intermittent (−)nicotine treatment. The present results showed that acute intermittent (−)nicotine treatment (four i.p. injections at intervals of 30 min), but not single injections, lead to a substantial and dose-related (0.1–2 mg/kg) up-regulation of fibroblast growth factor-2 messenger RNA levels in the cerebral cortex, in the hippocampus, in the striatum and ventral midbrain. This induction of fibroblast growth factor-2 expression peaked 4 h after the first injection and returned to normal levels within 24 h. The change of fibroblast growth factor-2 messenger RNA levels was associated with increased fibroblast growth factor-2 immunoreactivity mainly localized to nerve cells. The treatment was effective also when repeated in the same animals three or five days after the first injection. The pre-treatment with the non-competitive (−)nicotine receptor antagonist mecamylamine blocked the (−)nicotine effects on fibroblast growth factor-2 messenger RNA levels. In the above areas, no changes were observed in the fibroblast growth factor-1, 2 and 3 receptor messenger RNA levels nor in brain-derived neurotrophic factor messenger RNA levels.

The present data indicate an ability of intermittent (−)nicotine to increase fibroblast growth factor-2 in many tel- and diencephalic areas. In view of the trophic function of fibroblast growth factor-2, the previously observed neuroprotective effects of (−)nicotine may at least in part involve an activation of the neuronal fibroblast growth factor-2 signalling, and open up new avenues for treatment of Parkinson's disease and Alzheimer's disease based on the existence of nicotinic receptor subtypes enhancing fibroblast growth factor-2 signalling in many regions of the tel- and diencephalon. © 1998 Published by Elsevier Science Ltd.

Key words: basic fibroblast growth factors, gene expression, nicotine, nigrostriatal system, hippocampus, cerebral cortex.

The fibroblast growth factors (FGFs) are involved in various biological activities both *in vivo* and *in vitro*[3] and constitute a family of closely related polypeptides. Currently nine members of this family have been identified. The brain is one of the richest sources of mRNA encoding these proteins, that in turn are distributed in numerous CNS areas, both in neuronal and non-neuronal cells.[13,17,18,20,26,29,30,66,76] The FGFs exert their pleiotropic effect through specific high- and low-affinity cell surface receptors. The low-affinity receptors consist of various cell surface heparin sulphate proteoglycans.[42] To date, four distinct fibroblast growth factor high-affinity receptors (FGFRs) have been identified by molecular cloning methods, which have been named FGFR-1 or *flg*, FGFR-2 or *bek*, FGFR-3 and FGFR-4.[16,41,47,58,61] They all belong to the tyrosine kinase receptor family with three extracellular immunoglobulin-like domains and intracellular tyrosine kinase domains.[40]

Basic fibroblast growth factor (FGF-2) is one of the best characterized members of this family[3] and

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Abbreviations: BDNF, brain-derived neurotrophic factor; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DA, dopamine; EDTA, ethylenediaminetetra-acetate; FGF, fibroblast growth factor; FGF-2, basic fibroblast growth factor; FGFRs, fibroblast growth factor receptors; IR, immunoreactive, immunoreactivity; MK-801, dizocilpine maleate; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PLSD, Fisher's Protected Last Significant Difference; SSC, standard saline citrate.

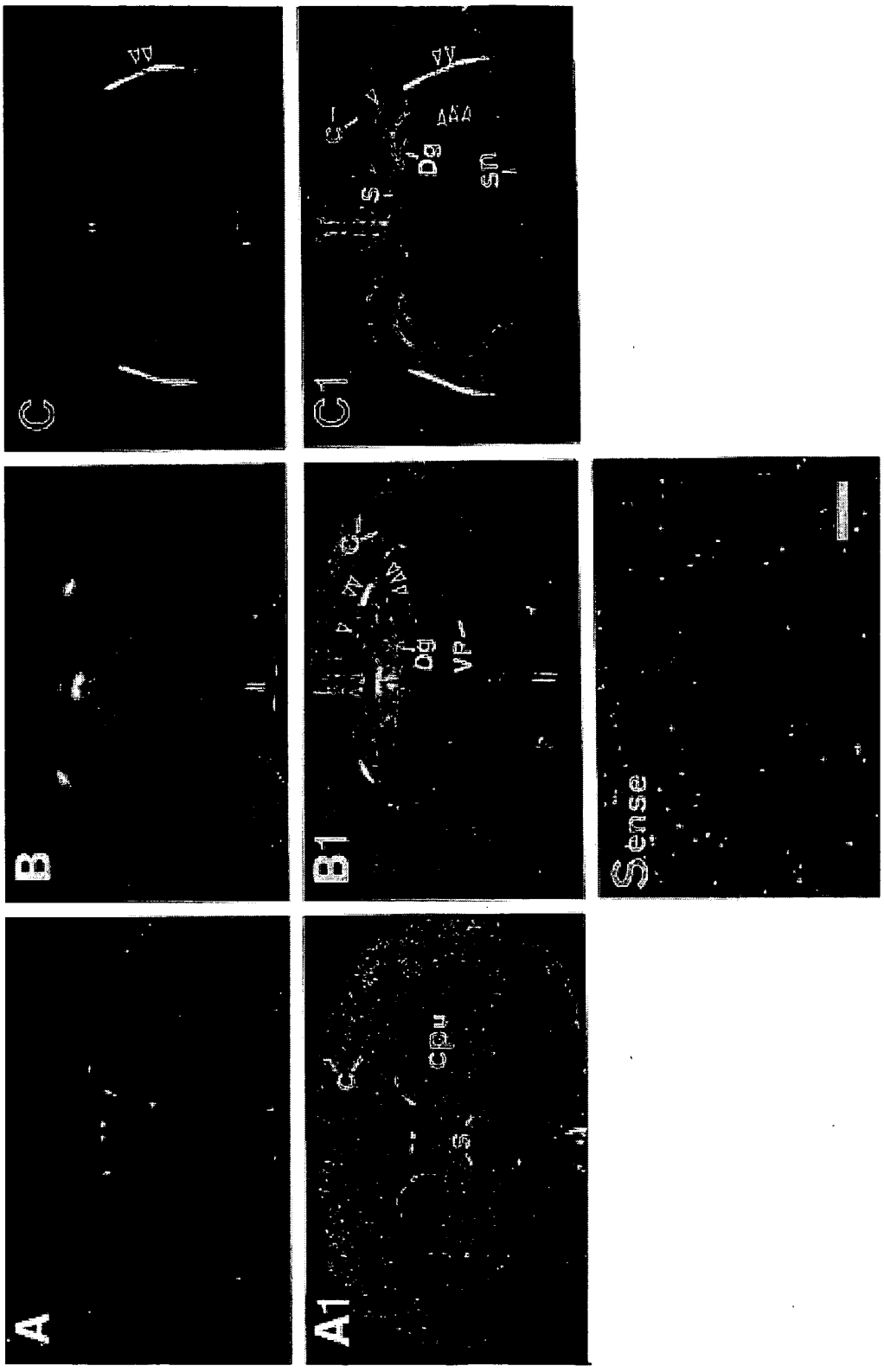


Fig. 1.

has been implicated to be important for the maturation and survival of dissociated neurons of several brain regions, including cerebral cortex,[52,72] hippocampus,[71] septum[45] and mesencephalon.[21,56] In addition, FGF-2 protects cultured central neurons from insults such as transient hypoglycemia[12] and *N*-methyl-D-aspartate (NMDA)-mediated excitotoxicity.[24,51] An *in vivo* protective role for FGF-2 has been indicated by studies showing that infusion of FGF-2 can support the survival of cholinergic basal forebrain neurons following fimbria transection, dopaminergic neurons following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity,[2,11,56] or hippocampal neurons following cerebral ischemia.[53] Further, neuroprotective functions of FGF-2 have been suggested by several observations on short- and long-lasting up-regulation of FGF-2 in different models of brain lesions, such as ischemia, seizures and trauma.[22,27,43,49,62]

Several studies have demonstrated a neuroprotective effect of nicotine treatment, such as in the nicotine-induced protection of cultured cortical neurons against NMDA receptor-mediated glutamate cytotoxicity.[1] Other studies have shown that chronic continuous infusion in combination with acute intermittent nicotine treatment can prevent dopamine (DA) cell loss following injury of dopaminergic neurons[25,35–39] Indirect trophic actions of nicotine are suggested from observations describing nicotine as a cognitive enhancer by increasing vigilance and improving learning and memory.[15,48] Based on these observations nicotinic drugs are considered as potential therapeutic agents in some neurological disorders such as Alzheimer's disease, in which they can be useful by slowing down memory deficits, and Parkinson's disease, in which a negative correlation has been observed between cigarette smoking and the incidence of disease.[5,32] Since the neurotrophic factors may be involved in many of the above reported experimental conditions showing neuroprotective effects of nicotine and, in general, in the above neurodegenerative disorders, it seems possible that nicotine may in part exert its neuroprotective actions by favouring the synthesis of neurotrophic factors.

The trophic function of FGF-2 in brain, and the widespread expression of FGF-2 mRNA and protein in brain regions together with the short- or long-lasting up-regulation of the FGF-2 gene after brain insults, suggested to us that FGF-2 could be one possible candidate among the neurotrophic factors involved in the nicotine-induced neuroprotective actions. Thus, to determine whether the reported neuroprotective effects of nicotine could be associated with an induction of neurotrophic factors in brain regions rich in high-affinity nicotine receptors, we examined the level of FGF-2 mRNA in brain regions of tel- and diencephalon of rats following single or acute intermittent nicotine treatment. We choose a single injection or an acute intermittent nicotine treatment, because previous results by Blum *et al.*[10] had shown that chronic continuous nicotine treatment down-regulates FGF-2 mRNA levels in the basal ganglia, probably related to a down-regulation of nicotinic receptors. In parallel, we also analysed the effects of nicotine on FGFR expression and on brain-derived neurotrophic factor (BDNF) expression, which like the FGF-2 is expressed in many brain regions[75] and is up-regulated after brain injury.[4]

EXPERIMENTAL PROCEDURES

Animals and (−) nicotine treatment

Specific pathogen-free adult Sprague–Dawley rats (B&K Universal Sweden) 200–250 g body weight were used in the present study. The rats were kept under controlled temperature and humidity conditions with standardized lighting (lights on at 6.00 a.m. and off at 8.00 p.m.) and had free access to food pellets and tap water. All animal experiments were approved by the local ethical committee (Stockholm Norra Försöksdjurs Etiska Kommittee'). Animals received, intraperitoneally, (i.p.), a single (−)nicotine injection or an acute intermittent (−)nicotine treatment or saline with a total of four injections given every 30 min. The time of killing for each experimental group was calculated from the first injection of (−)nicotine. The effects of (−)nicotine on FGF-2 mRNA levels were analysed by RNase protection assay and by *in situ* hybridization. Experiments on time course (4 h and 24 h) and dose–effect studies (doses ranging between 0.1–2 mg/kg) were performed, and each experimental group consisted of at least four animals.

Materials

(−)Nicotine hydrogen(+)tartrate (BDH Chemicals, Poole, U.K.). Mecamylamine dissolved in physiological saline was injected in a dose of 1 mg/kg i.p. 30 min before the (−)nicotine injection and repeated after 2 h. Dizocilpine maleate (MK-801), a non-competitive NMDA antagonist was injected (10 nmol/0.5 µl i.c.v.) 30 min before (−) nicotine treatment. The 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a non-NMDA receptor antagonist (Tocris Neuramin Essex, England), was dissolved in 1 N NaOH, pH adjusted to 7.4 with 0.2 N HCl followed by dilution with saline and injected 1 µg/0.5 µl i.c.v., 10 min before (−)nicotine treatment. The muscarinic receptor antagonist scopolamine dissolved in physiological saline was injected (10 mg/

Fig. 1. Photomicrograph of film autoradiograms of coronal brain sections through the striatum (A-A1), the dorsal hippocampus (B-B1) and the substantia nigra levels (C-C1) showing the expression of FGF-2 mRNA in control rats (A,B,C) and 4 h after acute intermittent (−)nicotine treatment with 1 mg/kg i.p. (A1,B1,C1). c, cerebral cortex; cpu, caudate–putamen; s, lateral septum in (A1); Dg, dentate gyrus; VP, ventral posterolateral and medial thalamic nuclei; s, subiculum in (C1); sn, substantia nigra; the one, two and three arrowheads indicate the CA1, CA2 and CA3 pyramidal layers, respectively. Sense shows a section hybridized with the corresponding FGF-2 sense RNA. Scale bar=1.5 mm.

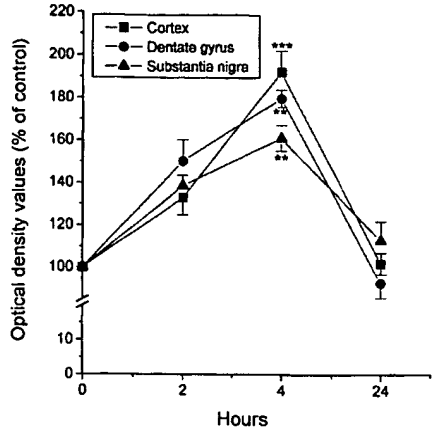


Fig. 2. Time course of FGF-2 mRNA expression in different brain regions of control rats and of rats 2 ,4 and 24 h after acute intermittent (−)nicotine treatment (1 mg/kg). Means ± S.E.M. (n=4–6) are shown as percent change of optical density values of the FGF-2 mRNA signals from film autoradiograms. 100% (cortex)=0.077 ± 0.006, ANOVA: $P<0.001$; 100% (dentate gyrus)=0.172 ± 0.02, ANOVA: $P<0.005$; 100% (substantia nigra) 0.067 ± 0.007, ANOVA: $P<0.01$. Cortex, dorsal cerebral cortex; substantia nigra, pars compacta and reticulata. PLSD: $^{**}P<0.01$; $^{***}P<0.001$ versus the respective control mean value.

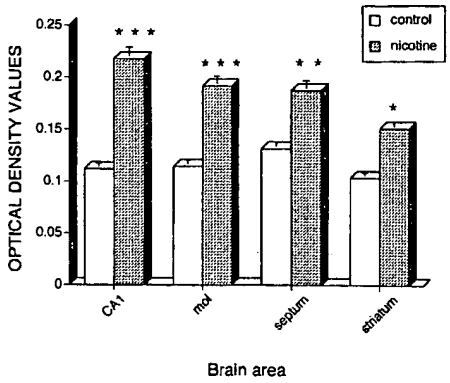


Fig. 3. Expression of FGF-2 mRNA in different brain regions of control rats and of rats 4 h after acute intermittent (−)nicotine treatment (1 mg/kg). Histograms on computerized image analysis of the optical density of the FGF-2 mRNA signals from film autoradiograms. Means ± S.E.M. (n=4–6). CA1, pyramidal cell layer of the hippocampus; mol, stratum moleculare of the dentate gyrus; septum, lateral septum; striatum, caudate-putamen. Mann–Whitney U-test: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus the respective control mean value.

kg i.p.) 20 min before (−)nicotine injection (Sigma, St Louis, MO). The efficacy of doses used for each antagonist has been reported previously.[7]

Quantitative analysis of fibroblast growth factor-2 messenger RNA by RNase protection assay

After the scheduled time, the rats were rapidly decapitated and the ventral midbrain and both the substantia nigra and the ventral tegmental area, the striatum, the hippocampus and the cerebral cortex (dorsal part of the hippocampus including the frontal and parietotemporal cortex) were rapidly dissected out, frozen and taken for FGF-2 mRNA analysis. RNA was isolated from the tissue samples and the levels of FGF-2 mRNA were determined by a quantitative ribonuclease protection assay as previously described by Blum.[9] For the nuclease protection assay a SmaI/XhoI fragment (nucleotides 525–1004) from a rat FGF-2 cDNA clone provided by Dr S. Shimasaki[65] which includes most of the coding region for the mature FGF-2 peptide, was subcloned into the vector Bluescript/SK$^+$ (Stratagene). Unlabelled sense and high specific activity ($\sim 1 \times 10^9$ c.p.m./μg) ^{32}P-labelled antisense RNA sequences were transcribed from the FGF-2/Bluescript subclone according to the manufacturer's recommendations. A standard curve was set up by known amounts of synthetic sense strand FGF-2 RNA ranging from 3 to 30 attomoles. The standards and known amounts of cytoplasmic RNA (5 μg) isolated from the tissue samples were hybridized with ~600 attomoles of FGF-2 antisense ^{32}P-labelled RNA probe for ~16 h at 68°C followed by S1 nuclease digestion (500–700 U; Pharmacia, Piscataway, NJ). Samples were then phenol-chloroform extracted, precipitated, reconstituted in TRIS-EDTA solution (100 mM Tris-HCl pH 2.6; 1 mM EDTA pH 8.0) and run on a 5% nondenaturing polyacrylamide gel. The gels were dried, exposed to a phosphorimaging screen and the amount of radioactivity in the protected RNA-RNA hybrids was counted. Attomoles of FGF-2 mRNA in the RNA samples were then determined by linear regression analysis of the standard curve.

In situ *hybridization probe preparation*

The FGF-2 RNA probe was synthesized by *in vitro* transcription from a FGF-2 fragment, amplified by polymerase chain reaction[49] and containing the promoter regions for SP6 RNA polymerase at the 3′ end and for T7 RNA polymerase at the 5′ end. The fragment represent the FGF-2-cDNA sequence between nucleotide 442 and nucleotide 660.[46] Transcription with T7 RNA polymerase resulted in a 218 nucleotide long sense RNA, whereas a 218 nucleotide long antisense RNA was obtained after *in vitro* transcription using SP6 RNA polymerase (Boehringer, Mannheim, FGR).

The preparation of FGFR-1 and FGFR-2 probes was made as previously reported by Orr-Urtreger *et al.*[55] The FGFR-1 cRNA probe was prepared from a 220 base pair long cDNA fragment cloned into the Sma site of pBluescript KS$^+$ (Stratagene, San Diego, CA), encoding the leader sequence and entering 24 nucleotides into the first immunoglobulin-like domain of the gene sequence reported by Safran *et al.*[63] The plasmid was linearized with PstI and used as template for T7 RNA polymerase to generate the antisense. The FGFR-2 cRNA probe was prepared from a 281 base pair cDNA fragment, starting 121 base pair upstream from the assumed initiator methionine and included the hydrophobic leader sequence and part of the first immunoglobulin-like loop of FGFR-2 gene sequence reported by Raz *et al.*[61] and cloned as described for the FGFR-1 probe. The antisense probes were generated using BamHI (T3). The FGFR-3 probe was prepared as reported by Peters *et al.*[60] and represented a 430 base pair cDNA fragment, encompassing nucleotides 1233–1663, encoding the transmembrane and juxtamembrane portions of FGFR-3, in the sequence reported by Ornitz and Leder[54] cloned into pBluescript KS$^+$, linearized with Hind III (T7) for antisense probe. The antisense cRNA probe detecting the BDNF transcripts was obtained by *in vitro* transcription from DNA fragment, coding for rat corresponding protein, cloned into pBSKS plasmid, and transcribed using T7 RNA polymerase.[67]

Labelling of RNA probes

The radiolabelling of the probes was performed as follows. The complementary DNA fragment of each probe

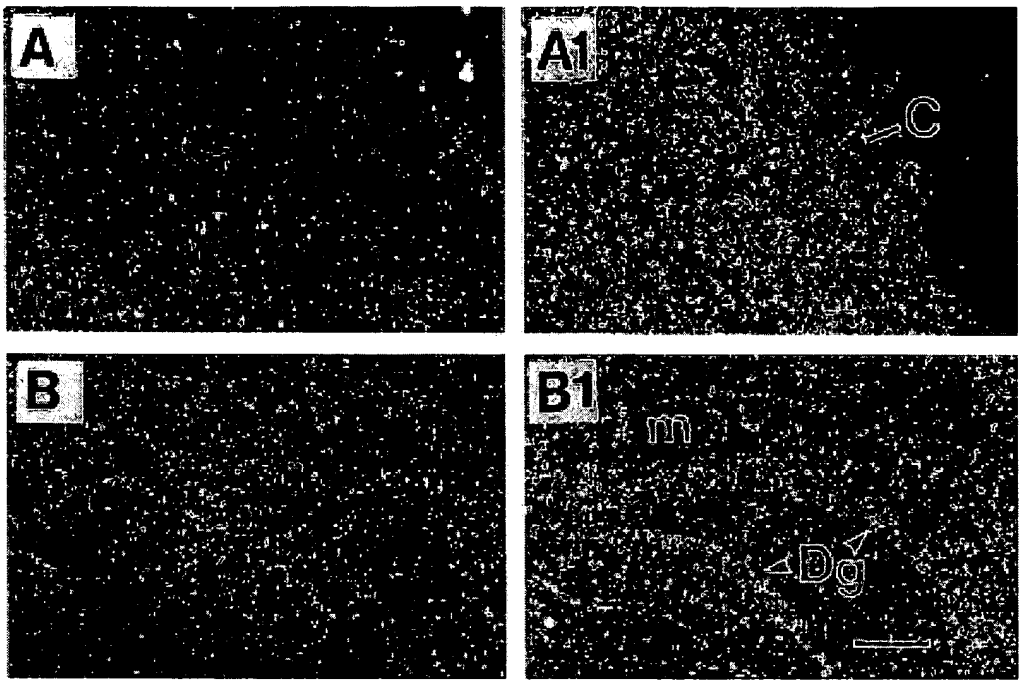


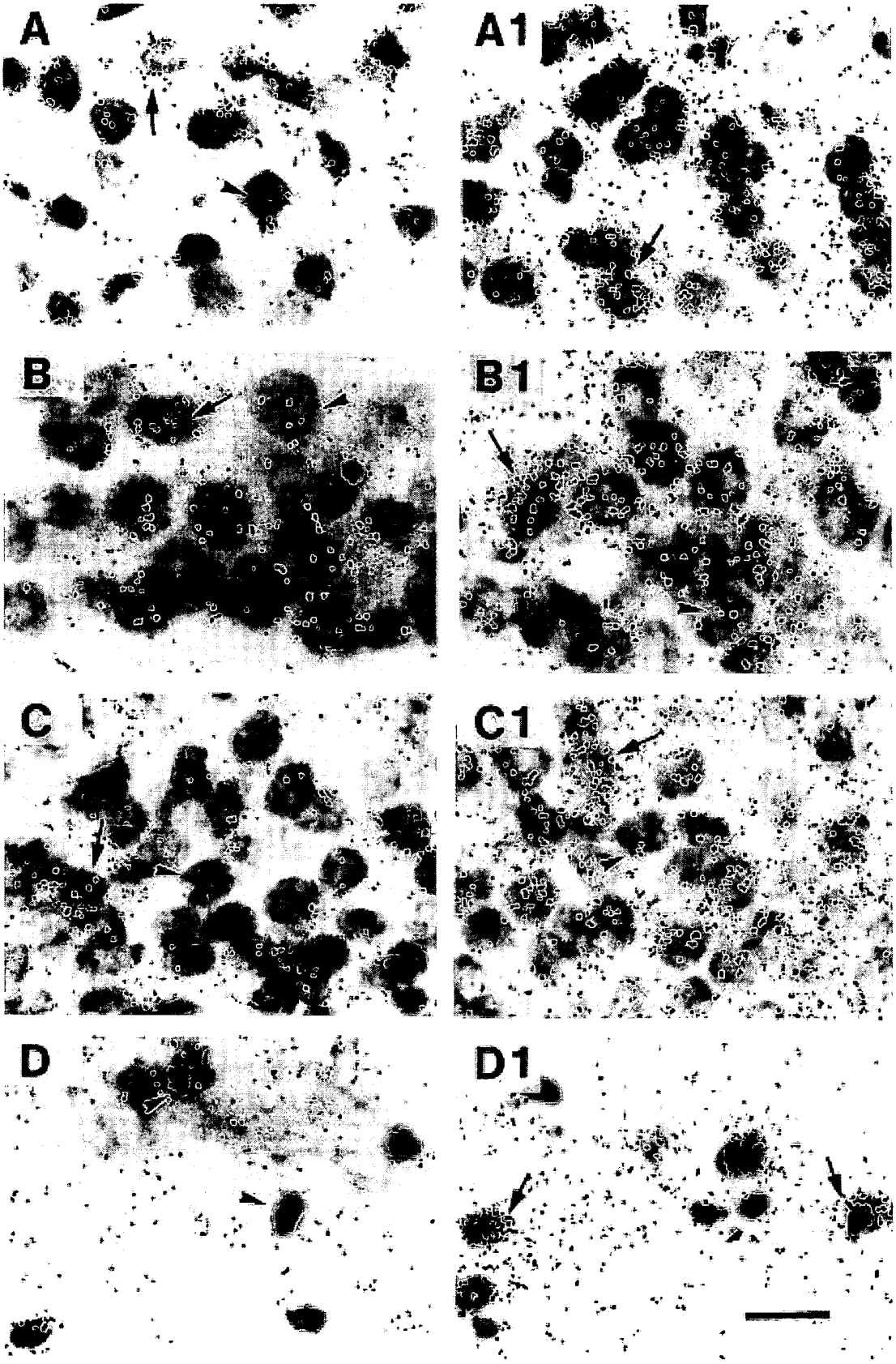
Fig. 4. Dark-field microautoradiographs from coronal sections showing labelling for FGF-2 mRNA in the cerebral cortex (A,A1) and the dentate gyrus of the hippocampus (B-B1). Note the low levels of FGF-2 mRNA in the control (A and B) and the increased levels of expression in the external layers of the cerebral cortex (A1) and in the dentate gyrus (B1) after 4 h of acute intermittent (−)nicotine treatment (1 mg/kg). c, cerebral cortex; m, stratum moleculare of the dentate gyrus; Dg, dentate gyrus. Scale bar=200 μm.

was incubated with a mixture of appropriate reagents: transcription buffer (40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine), with 12.5 nmol ATP, CTP and GTP, 500 pmol UTP and 125 pmol [^{35}S]α-UTP (Dupont NEN, Boston, MA, U.S.A.), 1 IU/μl RNAse inhibitor and 1 IU/μl of appropriate RNA polymerase at 37°C for 60 min. The cDNA template was digested by adding 20 ng/ml DNAse I at 37°C for 30 min. The transcripts were purified using Nensorb columns (DupontNEN, Boston, MA, U.S.A.) and analysed by denaturing formaldehyde gel electrophoresis.

In situ *hybridization procedure*

Serial coronal cryostat sections (14 μm) of rat brain at three different levels (striatal B: 1.20/0.20 mm, dorsal hippocampus B: −2.56/−3.60 mm and substantia nigra B: −4.80/−5.30) have been prepared based on the use of the atlas of Paxinos and Watson.[59] The *in situ* hybridization procedure was performed as follows. The slides were equilibrated at room temperature and the sections fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS) for 30 min. After washing with PBS, twice for 3 min the sections were deproteinated with 0.1 M HCl for 10 min. They were rinsed in PBS and acetylated with acetic anhydride 0.25% (pH 8.0) in 0.1 M triethanolamine for 20 min., rinsed in PBS and dehydrated in an ascending alcohol series and air-dried. Tissue sections were prehybridized with 250 μl prehybridization buffer (50% formamide, 50 mM Tris-HCl pH 7.6, 25 mM EDTA pH 8.0, 20 mM NaCl, 0.25 mg/ml yeast tRNA, 2.5 × Denhardt's solution) for 2 h at 52°C. After draining off the prehybridization buffer the sections were hybridized with hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 7.6, 5 mM EDTA pH 8.0, 10 mM sodium phosphate buffer, 0.2 mM dithiothreitol, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly-A-RNA, 10% dextran sulphate, 1 × Denhardt's solution). Each slide with four coronal sections, received 75 μl of hybridization buffer containing ^{35}S-labelled riboprobe (5×10^5 c.p.m.). We have found that using 5×10^5 c.p.m. of labelled riboprobe/slide optimizes the signal-to-background ratio for riboprobes using the present *in situ* hybridization protocol and post-hybridization washes described below. The sections were covered with coverslips. The hybridization was performed in a humidified chamber for 18 h at 52°C. After hybridization the slides were rinsed twice at 62°C in 1 × standard saline citrate (SSC) for 30 min, once in 50% formamide/0.5 × SSC for 30 min and rinsed again in 1 × SSC for 15 min. The sections were treated with 2 μg/ml RNAase A in RNAase buffer (0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA pH 8.0) for 30 min at 37°C. After two additional washings in 1 × SSC for 15 min at 62°C and one at room temperature, the sections were dehydrated in an ascending alcohol series and air-dried. For regional localization of mRNAs, hybridized sections were exposed for three weeks to β-Max Hyperfilm (Amersham), and subsequently coated with Kodak NTB-2 emulsion that was diluted 1:1 with distilled water, and stored in desiccated light-tight boxes at 4°C for six to eight weeks. Slides were developed in Kodak D-19, fixed in Kodak Rapid Fixer and counterstained with Cresyl Violet, dehydrated through graded alcohols, cleared in xylene, and coverslipped in DPX mountant.

The control of the hybridization method and the specificity of the cRNA riboprobes were performed using sense ^{35}S-labelled riboprobes, synthesized as described above. Semiquantitative data on mRNA levels were obtained by measuring the optical density values of labelling from the


A
A1
B
B1
C
C1
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film autoradiograms using a SAS Biovision image analysing system (Avanzati, Milan, Italy). The values for each region measured were defined as those obtained by subtracting the non-specific values (value in section incubated with labelled sense RNA probe minus background) from the total values (labelled sections with antisense RNA probe minus background).

Immunocytochemistry of fibroblast growth factor-2 immunoreactivity

Six rats were treated with saline and six with acute intermittent (−)nicotine at a dose of 1 mg/kg. After 8 h of the first injection of (−)nicotine the rats were perfused under deep barbiturate anaesthesia (pentobarbital, ACO, 60 mg/kg i.p.) with 0.9% saline followed by perfusion fixation with 0.16 M phosphate buffer (pH 6.9) containing 4% paraformaldehyde and 0.2% picric acid. Postfixation time was 90 min after which the brains were transferred into a 10% sucrose-containing phosphate buffer (0.1 M). 14 μm-thick sections were incubated with a mouse monoclonal antibody (bFM-1) raised against a synthetic 16 amino acid-long FGF-2 peptide (provided by Dr K. Nishikawa at the Kanazawa Medical University, Japan) and shown to recognize the conformation of the FGF-2 essential to biological activity[50] or a rabbit polyclonal antibody against an N-terminal (residues 1–24) synthetic peptide of bovine FGF-2[31] (generously provided by Dr A. Baird at the Whittier Institute for Diabetes and Endocrinology, Scripps Memorial Hospital, U.S.A.) overnight at 4°C in a moist chamber. These two antibodies can probably recognize different epitopes of the FGF-2 molecule which may help explain the different pattern of localization of FGF-2 immunoreactivity (IR), related e.g., to the different masking of parts of the FGF-2 molecule at different locations. The primary monoclonal and polyclonal antibodies were diluted 1:1500 and 1:1000 in PBS containing 0.3% Triton X-100, respectively. These antibodies do not cross-react with acidic FGF.[31,50] It was also confirmed by immunoblot analysis that the two anti-FGF-2 antisera used show little cross-reactivity with acidic FGF.[6,13] The sections were washed and incubated with a biotinylated antimouse or anti-rabbit antiserum (1:100) (Amersham, U.K.) for 2 h at room temperature. After two rinses in PBS at room temperature the sections were subsequently incubated with horseradish peroxidase–streptavidin complex (1:100) (Amersham, U.K.) for 1 h. The sections were then rinsed in Tris-HCl buffer (50 mM pH 7.4) and reacted in the same buffer containing 0.005% H_2O_2 and 0.02% diaminobenzidine. The sections were dehydrated and coverslipped with Entellan. In control experiments the sections were incubated with a FGF-2 antiserum which had been absorbed with 50 μg of the human recombinant FGF-2 (50 μg/ml antibody diluted 1:10).

Computer-assisted morphometry and microdensitometry

The monoclonal FGF-2 antibody only showed glial FGF-2-IR profiles, while the polyclonal FGF-2 antibody showed both neuronal and glial FGF-2-IR profiles. Image analysis of the IR obtained with the monoclonal antibody was used to measure the glial profiles and the polyclonal antibody was used to measure the neuronal profiles since the glial FGF-2-IR profiles could be removed by a skip function. Thus, a size threshold (circle diameter >6.5 μm) for accepting positive profiles was set. Neuronal FGF-2-IR profiles represent cytoplasmic profiles, while the astroglial profiles represent nuclear profiles. Together with the density of IR profiles also the specific IR area was determined which depends on the size and density of the FGF-2-IR profiles. The specific mean grey value of the FGF-2-IR profiles was determined by subtraction of the mean grey value of adjacent gray matter (unspecific value). This gives an index of the intensity (concentration of the antigen in the neuronal and glial FGF-2-IR profiles analysed). Pars compacta plus pars reticulata were analysed in the case of glial FGF-2-IR since it was difficult to establish the border between zona compacta and zona reticulata in this glial FGF-2 staining. Only pars compacta was analysed in the case of neuronal FGF-2 IR since the intention was to analyse mainly DA nerve cells. The area of specific IR profiles (spArea/mm^2) and the mean grey value of specific IR profiles (spMGV) are presented as means ± S.E.M. These procedures have been shown to produce reliable semiquantitative evaluations of the above parameters.[78]

Statistical analysis

The data were evaluated by one-way ANOVA with intergroup differences analysed by Fisher's Protected Last Significant Difference (PLSD) test, corrected by Bonferoni's procedure. For immunocytochemistry data analysis the Mann–Whitney U-test was used (non-parametrical procedures), since ratios are involved in evaluating the specific mean grey value, in combination with Bonferoni's correction for dependent samples.

RESULTS

Effects of (−)nicotine on regional fibroblast growth factor-2 messenger RNA levels

Time course

In situ hybridization. In agreement with previous observations,[18,27] the FGF-2 mRNA levels showed in control rats the typical pattern of labelling in the CA2 pyramidal layer of the hippocampus and a weak diffuse labelling within the other brain regions examined.

The analysis was performed at three rostrocaudal levels and the action of intermittent (−)nicotine is shown in Fig. 1, containing the brain regions also examined by RNase protection assay. The time course of (−)nicotine effects on FGF-2 mRNA levels in all the brain regions examined showed that significantly increased levels were reached 4 h after the (−)nicotine injection and returned to control levels within 24 h and the time courses of FGF-2 expression pattern in the dorsal cerebral cortex, the dentate

Fig. 5. Bright-field microautoradiographs showing FGF-2 mRNA-labelled cells in the splenium of the cerebral cortex (A-A1), in the CA1 pyramidal layer (B-B1), in the dentate gyrus (C-C1), and in the stratum lacunosum moleculare of the dentate gyrus (D-D1). Arrowheads indicate unlabelled cells and arrows indicate labelled cells. Note in the control rat (A–D) the low basal levels of FGF-2 mRNA with the labelling present only in scattered cells. Instead, in the acute intermittent (−)nicotine (1 mg/kg) treated rat (A1–D1) increased levels of FGF-2 mRNA are found both as increased grains per cell and as increased numbers of labelled cells. Note in A1–C1 the clusters of grains over cells with neuronal appearance, whereas in D1 (stratum lacunosum moleculare) the labelling is associated with small and strongly stained cells of a non-neuronal character. Scale bar=15 μm.

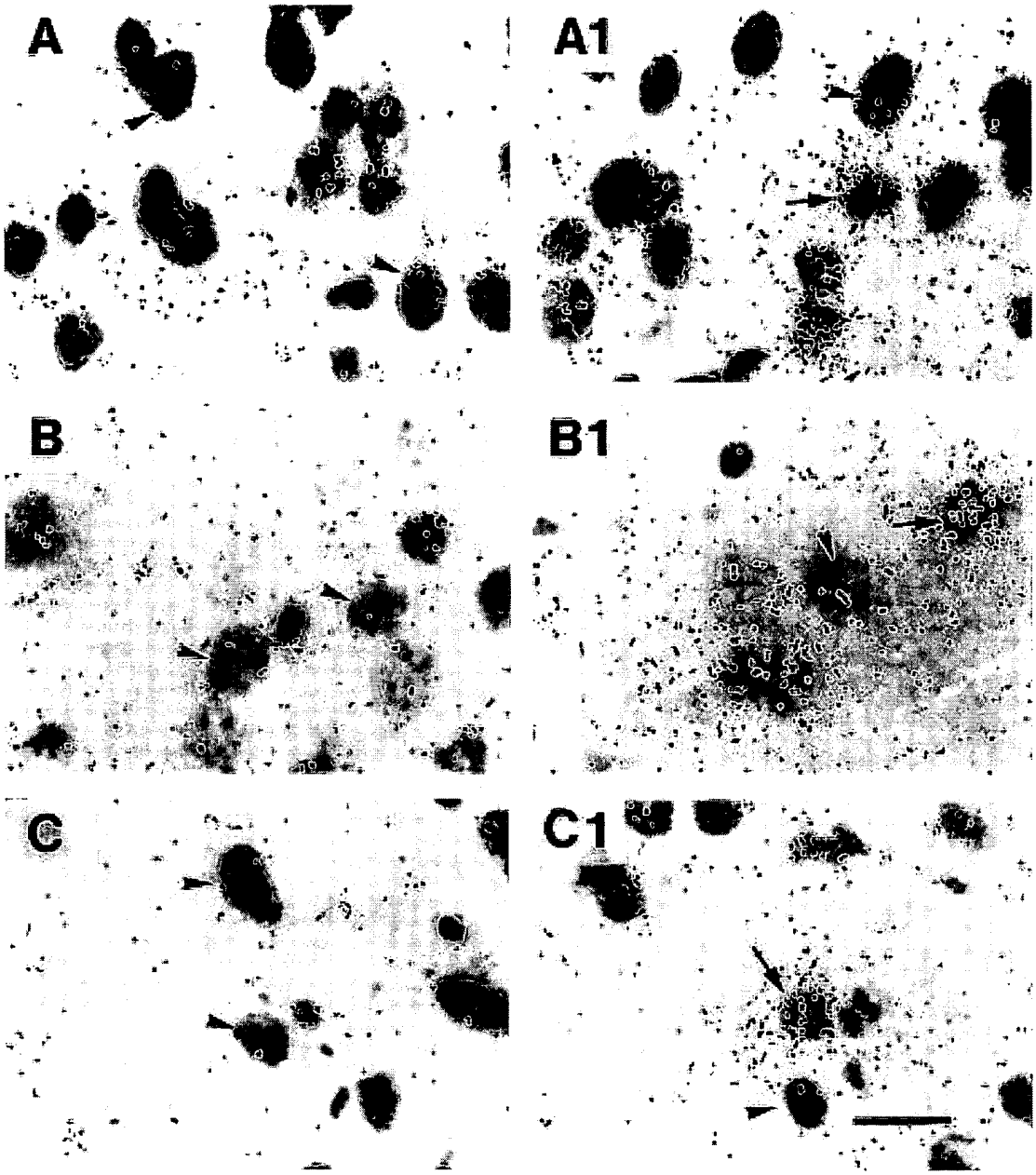

Fig. 6. Bright-field microautoradiographs showing FGF-2 mRNA-labelled cells in the cerebral cortex (A-A1), in the striatum (B-B1) and in the substantia nigra pars compacta (C-C1). Arrowheads indicate unlabelled cells and arrows indicate labelled cells. Note the undetectable basal levels of FGF-2 mRNA in controls (A–C), and increased levels of FGF-2 mRNA after acute intermittent (−)nicotine (1 mg/kg) treatment (A1–C1) in some large cell profiles. Scale bar=15 μm.

gyrus and the substantia nigra are reported (Fig. 2). In Fig. 3 the FGF-2 mRNA increase is shown 4 h after intermittent (−)nicotine injections in other regions of the hippocampus, such as the CA1 pyramidal cell layer and stratum moleculare, in the septum and in the striatum. Analysis of the regional and cellular localization of the FGF-2 mRNA increase 4 h after intermittent (−)nicotine injections further showed that: i) in the cerebral cortex (Fig. 4) higher levels of FGF-2 mRNA were expressed in the layers II–IV and in the layer II of the retrosplenial cortex; ii) in the perirhinal, piriform and

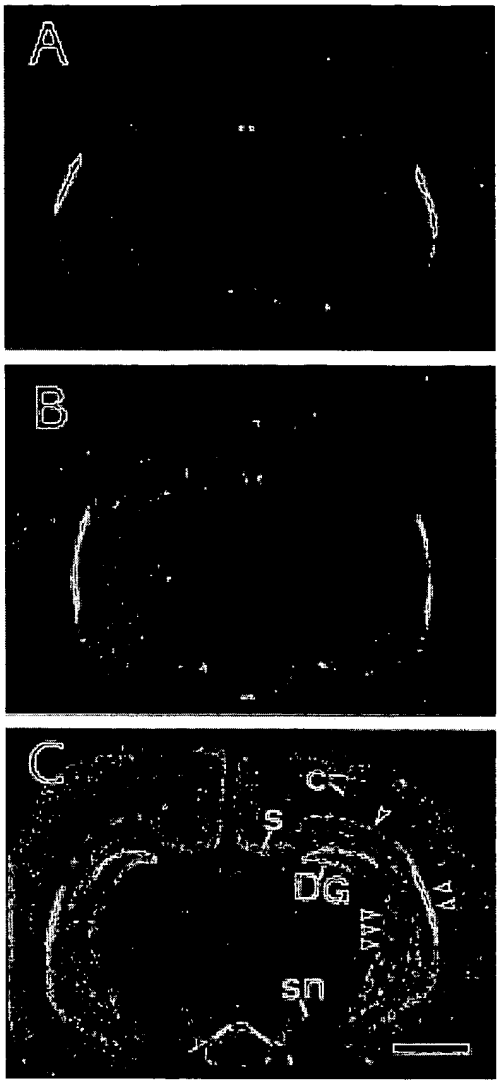

Fig. 7. Photomicrograph of film autoradiograms of coronal brain sections through the substantia nigra showing the expression of FGF-2 mRNA in a control rat (A), in a rat with acute intermittent (−)nicotine treatment (1 mg/kg) and killed after three days (B), and in a rat with acute intermittent (−)nicotine treatment repeated after three days and killed after 4 h of treatment (C). c, cerebral cortex; s, subiculum; Dg, dentate gyrus; sn, substantia nigra; one, two and three arrowheads indicate the CA1, CA2 and CA3 hippocampus pyramidal layers., respectively. Scale bar=1.5 mm.

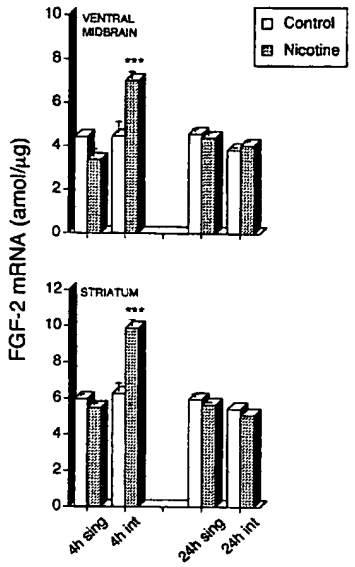

Fig. 8. Time course of effects of a single (−)nicotine injection (sing) or an acute intermittent (−)nicotine treatment (int) at a dose of 1 mg/kg on the FGF-2 mRNA levels in the ventral midbrain and striatum. FGF-2 mRNA was measured by a quantitative RNase protection assay. Means ± S.E.M. (n=4). Mann–Whitney U-test:: ***P<0.001 versus the respective control mean value.

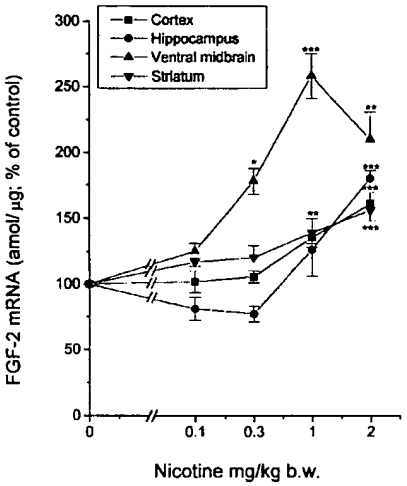

Fig. 9. Dose-effect of acute intermittent (−)nicotine treatment on the FGF-2 mRNA levels in different brain regions of rats killed after 4 h, percent changes are shown as means ± S.E.M. (n=6) of the FGF-2 mRNA (amol/µg), measured by a quantitative RNase protection assay. 100% (cortex)=6.9 ± 0.5, ANOVA: P<0.001; 100% (hippocampus)=8.1 ± 0.6, ANOVA: P<0.001; 100% (ventral midbrain)=3 ± 0.2, ANOVA: P<0.001; 100% (striatum)= 6.4 ± 0.5. PLSD: *P<0.05; **P<0.01; ***P<0.001 versus the respective control mean value.

entorhinal cortex FGF-2 mRNA expression was not up-regulated; iii) in the hippocampus (Fig. 4) the increased levels of FGF-2 expression belong to the nerve cells of the dentate gyrus, the pyramidal cell layers including the subiculum, and to non-neuronal cells as shown by the diffuse labelling within the stratum moleculare of the dentate gyrus, stratum lacunosum moleculare, radiatum and oriens of the hippocampus; iv) in other brain regions, such as the geniculate nuclei, the medial terminal nucleus accessory optic tract, and in the ventral posterolateral and

Table 1. Effects of pretreatment with mecamylamine or scopolamine on (−)nicotine-induced fibroblast growth factor-2 messenger RNA expression

Treatment	Brain areas		
	Cerebral cortex	Dentate gyrus	Substantia nigra
(6) Control saline (i.p.)	0.098 ± 0.04	0.207 ± 0.18	0.092 ± 0.006
(4) Nicotine (Nic)	0.191 ± 0.014	0.371 ± 0.021	0.126 ± 0.007
	$P<0.001$	$P<0.001$	$P<0.01$
(3) Mecamylamine (single dose)	0.092 ± 0.019	0.205 ± 0.015	0.097 ± 0.012
	N.S.	N.S.	N.S.
(3) Mecamylamine (repeated dose)	0.090 ± 0.005	0.216 ± 0.033	0.105 ± 0.009
	N.S.	N.S.	N.S.
(4) Mecamylamine+Nic	0.103+0.022	0.212 ± 0.012	0.086 ± 0.012
	N.S.	N.S.	N.S.
(3) Scopolamine+Nic	0.203 ± 0.014	0.342 ± 0.036	0.136 ± 0.016
	$P<0.001$	$P<0.01$	$P<0.01$

Computerized image analysis showing optic density of FGF-2 mRNA levels from audioradiograms. Mean ± S.E.M. ANOVA: cerebral cortex, $P<0.001$; dentate gyrus, $P<0.001$; substantia nigra, $P<0.02$. In brackets are indicated the number of animals used. P values: PLSD with Bonferoni's procedure, versus control mean value.

Table 2. Effects of pretreatment with dizocilpine maleate or 6-cyano-7-nitroquinoxaline-2,3-dione on (−)nicotine-induced fibroblast growth factor-2 messenger RNA expression

Treatment	Brain areas		
	Cerebral cortex	Dentate gyrus	Substantia nigra
(6) Control saline (i.c.v.)	0.0987 ± 0.006	0.215 ± 0.016	0.096 ± 0.01
(4) Nicotine (Nic)	0.192 ± 0.04	0.374 ± 0.022	0.140 ± 0.016
	$P<0.001$	$P<0.01$	$P<0.05$
(3) MK801+Nic	0.194 ± 0.011	0.362 ± 0.039	0.139 ± 0.013
	$P<0.001$	$P<0.01$	$P<0.05$
(3) CNQX+Nic	0.180 ± 0.022	0.374 ± 0.015	0.144 ± 0.013
	$P<0.01$	$P<0.01$	$P<0.05$

Computerized image analysis showing optic density of FGF-2 mRNA levels from audioradiograms. Mean ± S.E.M. ANOVA: cerebral cortex, $P<0.001$; dentate gyrus, $P=0.001$; substantia nigra, $P<0.02$. In brackets are indicated the number of animals used. P values: PLSD with Bonferoni's procedure, versus control mean value.

medial thalamic nuclei, the FGF-2 expression was also increased (data not shown); v) in the substantia nigra both the pars reticulata and compacta showed increased levels of FGF-2 mRNA.

The autoradiographic analysis demonstrated that the increased levels are predominantly expressed in neuronal cell profiles (Figs 5, 6).

It was also analysed if the acute intermittent (−)nicotine treatment could be effective when repeated in the same animals three days or five days after the first injections. The repeated intermittent administration of (−)nicotine gave a similar induction pattern of FGF-2 expression as after the first intermittent treatment (Fig. 7). Animals, which received only one acute intermittent treatment with nicotine and were killed three days or five days later, did not show changes in FGF-2 mRNA levels compared to saline-treated rats (Fig. 7).

RNAse protection assay. Time-dependent changes of FGF-2 mRNA level were studied at 4 h and 24 h after acute intermittent injections of (−)nicotine (1 mg/kg i.p.). The treatment, as shown in Fig. 8, revealed a significant increase both in ventral midbrain and striatum 4 h after the (−)nicotine injections, returning to control levels by 24 h post-injection. The time course studies performed to characterize the effect of a single (−)nicotine injection failed to show changes in the levels of FGF-2 mRNA (Fig. 8).

Dose–effect curve by RNase protection assay. Based on the time course analysis, the time point of 4 h after the acute intermittent (−)nicotine treatment was selected (Fig. 9). In the ventral midbrain the FGF-2 mRNA levels began to increase with the dose of 0.1 mg/kg, and reached the highest level with the dose of 1 mg/kg. In the striatum the FGF-2 mRNA levels were dose-dependently increased with maximal action at the dose of 2 mg/kg. In the hippocampus, the levels of FGF-2 mRNA were substantially increased at 1 mg/kg and further increased at 2 mg/kg, but showed unexpectedly a trend for a decreased level at lower doses. The cerebral cortex area

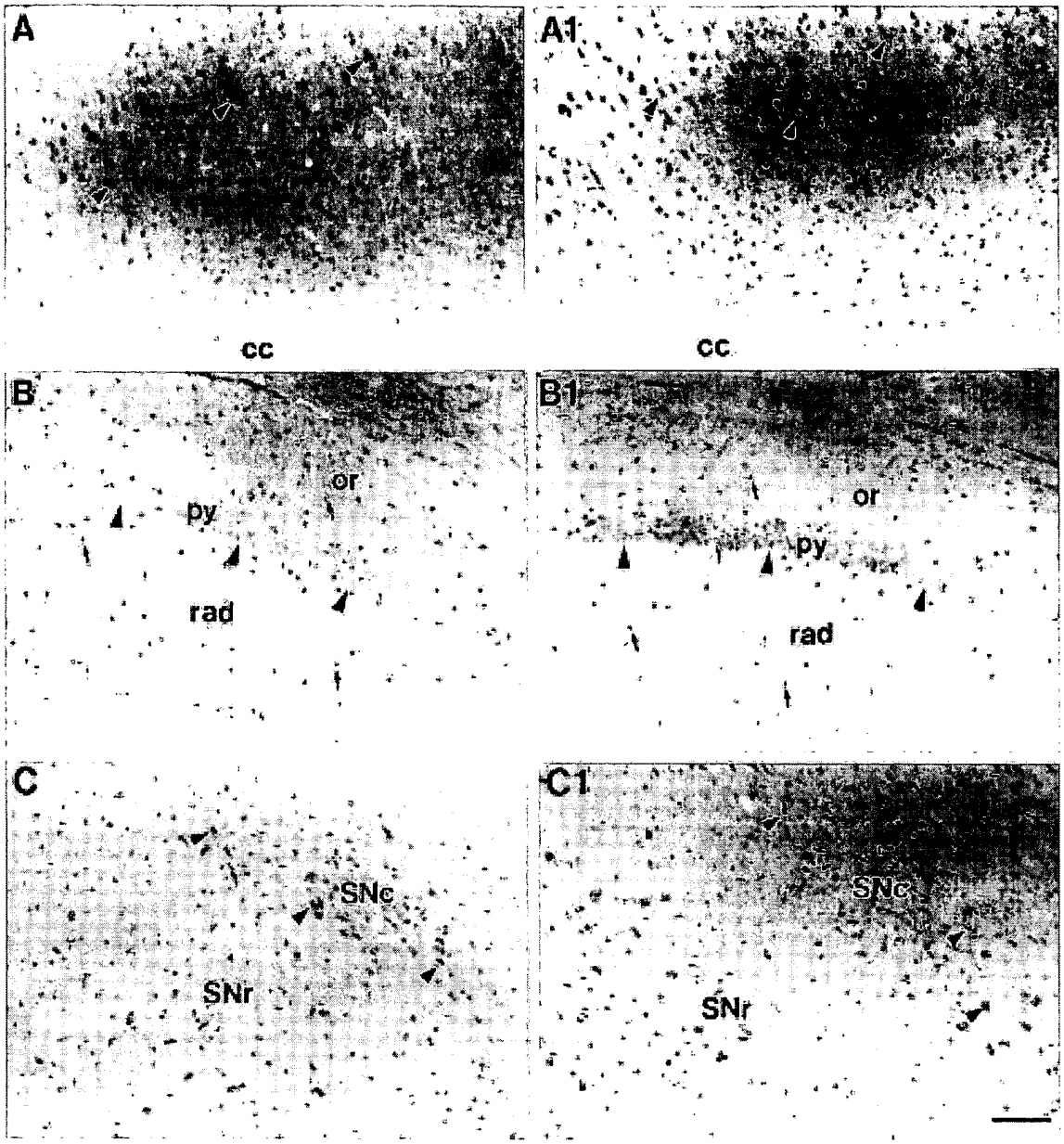

Fig. 10. The microphotographs show FGF-2-IR in a coronal sections in control rat (A–C) and in a rat 8 h after acute intermittent (−)nicotine treatment (A1–C1). cc, corpus callosum; py, pyramidal layers of the hippocampus; rad, stratum radiatum; or, stratum oriens; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; The arrowheads in A-A1 and C-C1 point to neuronal FGF-2-IR, and in B-B1 to the CA1 pyramidal layer of the hippocampus. The arrows in B-B1 point to FGF-2-IR in small glial cells of the stratum radiatum and oriens of the hippocampus. Scale bar=100 μm.

examined corresponds to the parietal and frontal cortex, dorsal to the hippocampus, including part of the occipital cortex. FGF-2 mRNA levels in this cortical area were dose-dependently increased in a similar way as found in the striatum.

Effects of pretreatment with mecamylamine. Mecamylamine, a non-competitive nicotinic receptor antagonist, was used to examine the specificity of the (−)nicotine effects. Pretreatment with mecamylamine was made 30 min before the (−)nico-

Table 3. Effects of acute intermittent (−)nicotine treatment on microdensitometric and morphometric parameters of the basic fibroblast growth factor immunoreactive neuronal profiles in CA1 pyramidal layer of the hippocampus, in the II–VI layers of cerebral cortex and in the substantia nigra pars compacta

Region	Treatment	*n*	spMGV	Area (μm²/mm²)
CA1 pyramidal layer	Control	6	42 ± 4.7	84543 ± 5345
	Nicotine	5	50 ± 4.9	142804 ± 15628
			N.S.	$P<0.01$
Frontoparietal cortex	Control	6	47 ± 3	14437 ± 3042
	Nicotine	5	57 ± 2.9	33458 ± 7724
			N.S.	$P<0.05$
Substantia nigra pars compacta	Control	6	50 ± 8	48922 ± 3523
	Nicotine	6	79 ± 3.4	85670 ± 8825
			$P<0.05$	$P<0.05$

For details on treatment, see Experimental Procedures. Mean ± S.E.M. are shown. The unpaired Mann–Whitney *U*-test was used for the statistical analysis in combination with Bonferoni correction for dependent samples. *n*, number of rats; Area, specific immunoreactive area; spMGV, specific mean grey value.

Table 4. Effects of acute intermittent (−)nicotine treatment on microdensitometric and morphometric parameters of the basic fibroblast growth factor immunoreactive astroglial profiles in stratum radiatum of the hippocampus, in the II–VI layers of cerebral cortex and in the substantia nigra pars reticulata and compacta

Region	Treatment	*n*	spMGV	Area (μm²/mm²)
Stratum radiatum	Control	5	56 ± 6.9	7257 ± 643
	Nicotine	5	59 ± 5.6	7710 ± 716
			N.S.	N.S.
Frontoparietal cortex	Control	6	50 ± 2.2	8687 ± 499
	Nicotine	6	49 ± 2.4	8908 ± 598
			N.S.	N.S.
Substantia nigra pars reticulata and compacta	Control	6	43 ± 2.1	12533 ± 1167
	Nicotine	6	46 ± 3.2	13280 ± 1038
			N.S.	N.S.

For details on treatment, see Experimental Procedure. Mean ± S.E.M. are shown. The unpaired Mann–Whitney *U*-test was used for the statistical analysis in combination with Bonferoni correction for dependent samples. Area, specific immunoreactive area; spMGV, specific mean grey value.

tine injection in a dose of 1 mg/kg given i.p. and repeated after 2 h. In this model the study of FGF-2 mRNA expression 4 h after the (−)nicotine injection revealed a complete block of (−)nicotine actions by mecamylamine on FGF-2 mRNA levels in all the brain regions examined (Table 1). Treatment with mecamylamine alone, also when repeated after 5 h and 10 h and killing of the rats 12 h after the first injection, did not change FGF-2 expression (Table 1).

Effects of pretreatment with scopolamine, dizocilpine maleate and 6-cyano-7-nitroquinoxaline-2,3-dione. Several studies on presynaptic nicotinic receptors have shown that nicotine facilitates in different brain regions the release of various neurotransmitters including excitatory neurotransmitters such as acetylcholine and glutamate.[69,77] Since it has been shown that glutamatergic agonists can induce FGF-2 expression[27,62] the effects of (−)nicotine treatment on FGF-2 mRNA levels were evaluated in rats pretreated with the NMDA antagonist MK-801 or the non-NMDA antagonist CNQX. No attenuation in the up-regulation of FGF-2 mRNA levels by (−)nicotine treatment was found (Table 2). Similarly, pretreatment with the cholinergic muscarinic anatgonist scopolamine failed to counteract the effect of (−)nicotine on FGF-2 expression (Table 1).

Effects of nicotine on regional fibroblast growth factor-2 immunoreactivity

Immunocytochemistry. As previously reported,[26] the neuronal IR was predominantly cytoplasmic and restricted to the perikarya and the larger dendritic proximal processes. As seen in (Fig. 10; Table 3), acute intermittent (−)nicotine treatment after 8 h in a dose of 1 mg/kg significantly increased the specific IR area in the CA1 of the hippocampus and in the zona compacta of the substantia nigra, but the increase in the SpMGV instead was only significant in the substantia nigra. However, in the frontoparietal cortex the increase in the specific IR area did not reach significance probably due to increased

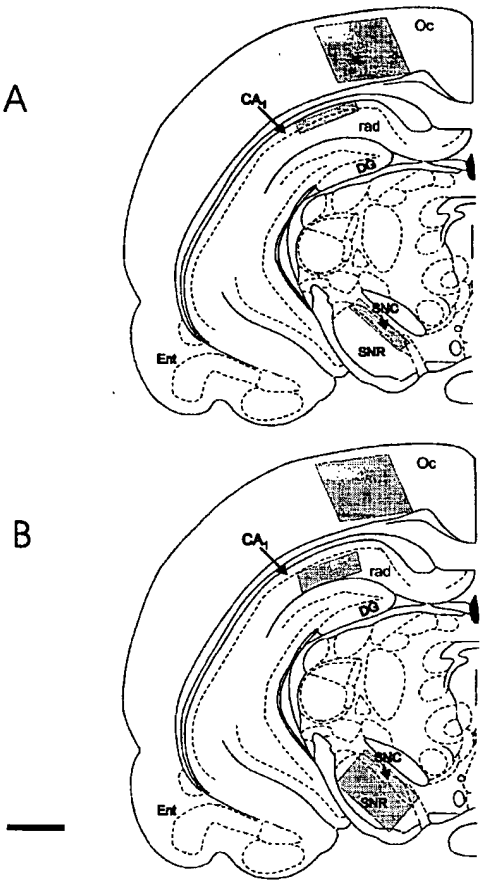

Fig. 11. Schematic representation of choronal atlas section (Paxinos and Watson) through the substantia nigra (Bregma = −5.30) used to illustrate the position of the sampled areas to measure the density of FGF-2-IR profiles for nerve cells (A) and glial cells (B). SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; Oc, occipital cortex; DG, dentate gyrus; CA1 pyramidal cell layer; rad, stratum radiatum of the hippocampus; Ent, entorhinal cortex. Scale bar=0.8 mm

variability. By contrast, astroglial FGF-2-IR was not changed in the same brain regions (Table 4). The sampled field for measuring the density and intensity of IR profiles correspond to the areas shown in Fig. 11. No immunostaining with the two FGF-2 antisera was found after incubation of the antibodies with human recombinant FGF-2.

Effects of nicotine on fibroblast growth factor receptor-1, 2 and 3 messenger RNA levels

In situ *hybridization.* The normal distribution of hybridization to FGFR mRNAs obtained in the present work is in agreement with other reports[30,73] and our previous data.[8] The (−)nicotine treatment, at the doses and time intervals studied, did not change the basal mRNA levels of FGFRs in the brain regions examined (Fig. 12).

Effects of nicotine on regional brain-derived neurotrophic factor messenger RNA levels

In situ *hybridization.* To determine whether in general genes encoding neurotrophic factors could be influenced by (−)nicotine treatment we choose to study the gene expression of BDNF since it is expressed in many brain regions examined in the present work and can be induced by e.g., pharmacological treatments or brain injury,[4] as is the case with FGF-2. The normal distribution of BDNF mRNA hybridization observed in the present work is in agreement with other reports.[75]

The acute intermittent (−)nicotine treatment, at the dose of 1 mg/kg after 4 h, did not change the basal mRNA levels of BDNF in any brain region examined (Fig. 13).

DISCUSSION

Acute intermittent treatment with (−)nicotine transiently and markedly increased FGF-2 mRNA in large parts of the tel- and diencephalon predominantly located to nerve cell body profiles, in the substantia nigra, in the hippocampal pyramidal cell layers, in the cerebral cortex, and in the striatum. The parallel increase of FGF-2-IR in some brain regions temporally followed the increase in neuronal FGF-2 mRNA. In fact, a significant increase of FGF-2 protein levels was observed at 8 h, suggesting that it is an event secondary to the elevation of FGF-2 gene expression which peaks after 4 h of (−)nicotine treatment.

Relationship to neurotrophic actions of nicotine

Acute intermittent nicotine treatment in combination with chronic continuous infusion has been shown to produce partial neuroprotection against mechanical injury to DA pathways, based on morphological, neurochemical and physiological findings.[25,35,37,39] This type of treatment, involving the acute intermittent treatment in combination with a two week continuous administration of (−)nicotine via Alzet minipumps, has also been shown to partly protect against MPTP-induced degeneration of the nigrostriatal DA neurons in the black mouse.[36,38] Subsequent work demonstrated that it was the acute intermittent treatment with (−)nicotine in relation to the MPTP treatment that was responsible for the neuroprotective activity of (−)nicotine.[37] In contrast, the continuos administration of (−)nicotine alone over two week period led to a marked enhancement of the MPTP-induced degeneration of the nigrostriatal DA system in the black mouse.[37] The present demonstration that acute intermittent treatment with (−)nicotine produced substantial

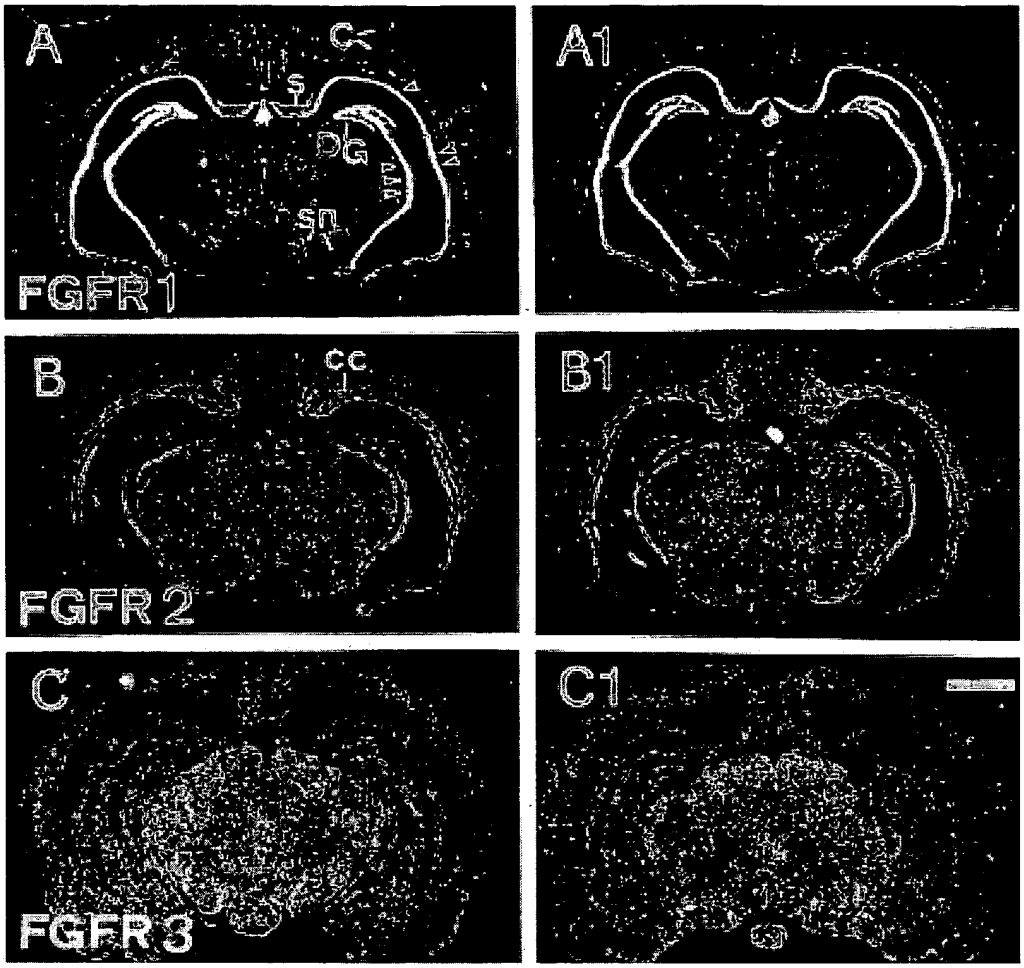

Fig. 12. Photomicrograph of film autoradiograms of coronal brain sections through the substantia nigra showing the levels of FGFR-1 (A,A1), FGFR-2 (B,B1) and FGFR-3 (C,C1) mRNAs in a control rat (A–C) and in a rat with acute intermittent (−)nicotine treatment (1 mg/kg) and killed after 4 h (A1–C1). C, cerebral cortex; DG, dentate gyrus; s, subiculum; sn, substantia nigra; cc, corpus callosum; one, two and three arrowheads indicate the CA1, CA2 and CA3 hippocampus pyramidal layers., respectively. Scale bar=1.5 mm.

increases in FGF-2 mRNA levels in the tel- and diencephalon offers a new explanation of the neuroprotective activity of acute intermittent (−)nicotine treatment against MPTP and mechanical injury of the DA neurons. Thus, enhancement, particularly of neuronal FGF-2 mechanisms, may underlie at least in part this neuroprotective activity. Such actions by (−)nicotine on FGF-2 mechanisms may also help to explain the negative correlation found between cigarette smoking and incidence of Parkinson's disease.[5]

The fact that continuous administration of (−)nicotine alone over two weeks led to a marked increase in MPTP-induced neurotoxicity of the DA pathways in the black mouse may also have an explanation from our recent experiments on the effects of such treatments on FGF-2 mRNA levels in brain of normal rats. Thus, chronic continuous (−)nicotine treatment, made possible by minipump implantation, significantly and dose-dependently reduced FGF-2 mRNA levels within the neostriatum and the substantia nigra.[10] Such a down-regulation of FGF-2 mRNA levels in the basal ganglia may be related to the desensitization of the nicotinic receptors known to occur after prolonged exposure of these receptors to nicotinic agonists producing a progressive decline of the response amplitude of these receptors.[28] Also *in vitro*, nicotine has been shown to protect cultured neurons from cerebral cortex against insults, such as NMDA receptor-mediated glutamate cytotoxicity,[1] and in the present data we report increased levels of FGF-2 mRNA in the cerebral cortex. Finally the increased levels of FGF-2 mRNA in the hippocampus could help explain the effects of

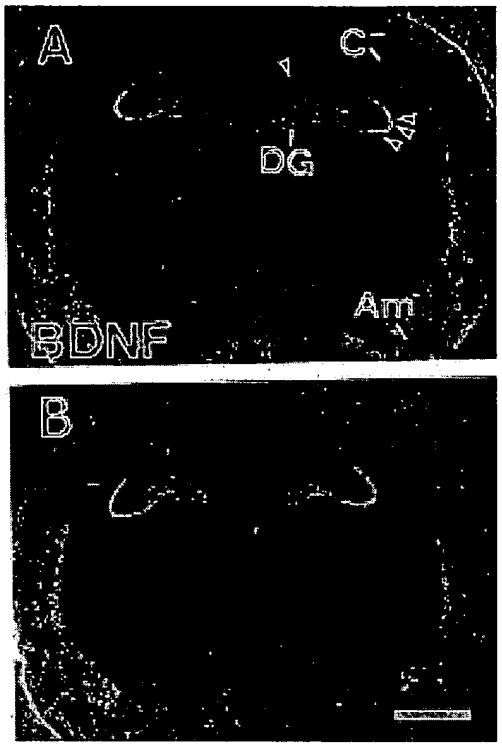

Fig. 13. Photomicrograph of film autoradiograms of coronal brain sections through the dorsal hippocampus showing the expression of BDNF mRNA in a control rat (A) and in a rat with acute intermittent (−)nicotine treatment (1 mg/kg) and killed after 4 h (B). C, cerebral cortex; DG, dentate gyrus; Am, amygdala; one and three arrowheads indicate the CA1 and CA3 hippocampus pyramidal layers., respectively. Scale bar=1.5 mm.

nicotine on cognitive functions, such as learning and memory.[15,48]

Possible correlation of the distribution pattern of areas showing increased fibroblast growth factor-2 expression with that of high-affinity [^{3}H]nicotine binding sites

Nicotine-induced effects on FGF-2 expression were antagonized by the non-competitive nicotine receptor antagonist mecamylamine, indicating an involvement of nicotine receptors. In contrast, blockade of NMDA and non-NMDA receptors as well as of muscarinic receptors with relevant doses of MK-801, CNQX and scopolamine, respectively, failed to block the (−)nicotine-induced increase of FGF-2 mRNA levels. It remains to be determined which nicotine receptor subtypes[28] are involved in this action. Nevertheless, it was possible to examine the existence of a correlation between the distribution of specific nicotine high-affinity binding sites from previously published data[14,34,48,64,70] and the distribution of brain regions showing an up-regulation of FGF-2 mRNA levels after the acute intermittent (−)nicotine treatment. A positive correlation was found suggesting that an α4β2 nicotinic receptor may be responsible for inducing the (−)nicotine actions on FGF-2 gene expression, since this nicotinic receptor is responsible for high-affinity nicotine binding in rat brain.[15] However, in some brain regions, such as several thalamic nuclei, that show high densities of high-affinity nicotine receptors, FGF-2 expression was not markedly induced by (−)nicotine.

Nicotinic receptor activation and gene regulation

The ability of nicotinic receptor activation to influence gene expression has been shown for early genes, such as *c-fos*, *c-jun* and *jun-B*, and late onset genes, such as proenkephalin A and tyrosine hydroxylase.[33,44,57,74] It is unknown which early gene activation contributes to the presently observed increases of FGF-2 expression found after (−)nicotine. An important intracellular signal for these events may be an increased Ca^{2+} influx through the nicotinic receptor-linked ionic channels. Previous results from the hippocampus have shown that the nicotinic antagonist, α-bungarotoxin, increases mRNA levels for BDNF and nerve growth factor, suggesting a negative regulation by α-bungarotoxin-sensitive nicotinic receptors of BDNF and nerve growth factor.[23] In the present model of (−)nicotine treatment no changes of BDNF mRNA expression were shown in the brain regions analysed including the hippocampus.

Comparison of nicotine actions on fibroblast growth factor-2 expression to other models with increased fibroblast growth factor-2 gene regulation

The temporal and spatial pattern of FGF-2 induction in the brain observed after acute intermittent (−)nicotine treatment appears different compared to that found after other challenges leading to FGF-2 induction. Thus, the FGF-2 mRNA increase following convulsive seizures,[62] peaked at 10 h, and was also observed in the entorhinal cortex, but not in the striatum, in contrast to the present findings. Similarly, recurrent limbic seizure-induced FGF-2 mRNA levels,[27] peaked at 12 h, was still high after 24 h, and was also observed in brain regions like the entorhinal cortex and the amygdala, where no actions were found after intermittent (−)nicotine treatment. An increased level of FGFR-1 and 2 mRNA has been reported after brain injury or ischemia,[19,68] whereas we found no change after nicotine treatment. Induction of FGF-2 mRNA level by mechanical trauma or ischemia[43,49] seems to take place exclusively in reactive astrocytes and to exist for several days. In contrast the increased FGF-2 mRNA levels seen after intermittent (−)nicotine predominantly exist within nerve cells and disappeared within 24 h. The above spatiotemporal

differences further suggest a specific action of this nicotinic receptor subtype in increasing regional brain FGF-2 mRNA and protein levels.

CONCLUSION

In conclusion, the present data showed a defined temporal and regional pattern of FGF-2 mRNA and protein induction after acute intermittent nicotine treatment and suggest, in view of the trophic function of FGF-2, that the previously observed neuroprotective effects of nicotine may at least in part involve an activation of the neuronal FGF-2 signalling. This implication becomes especially strong in view of the demonstration that acute intermittent (−)nicotine treatment can repeatedly induce FGF-2 mRNA levels. These results open up new avenues for treatment of neurodegenerative disease based on the existence of nicotinic receptor subtypes enhancing FGF-2 signalling in many regions of the tel- and diencephalon.

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